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14. ABSTRACT
We have successfully prepared sense expression libraries, characterized the size of their inserts which are fused to a green fluorescent protein tag, and packaged these libraries into lentivirus. Four different human MDS bone marrow isolates have been transfected with lentivirus at multiplicity of infection of 0.1. A collection of 250 puromycin clones were isolated, genomic DNA was prepared, and clones were screened for ability to undergo myeloid differentiation in response to GM-CSF. Only ~30% of the puromycin resistant clones (80) acquired this phenotype. Transfection of MDS cells with antisense libraries did not generate any clones that acquired the desired (differentiative) phenotype. PCR was used to identify presence of the trans gene in the 80 clones. DNA sequencing is currently being performed to identify the inserts. Lentivirus is being prepared to express these inserts in naïve MDS cell pools. This validation will ensure that the insert causes the desired phenotype.

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Myelodysplastic syndrome, lentivirus, cDNA libraries, complementation

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Introduction

Rationale: The focus of our proposal is the identification of normal human genes, that when overexpressed by transduction of a lentiviral vector, complement the underlying genetic defect in MDS cells. Our alternate approach was to downregulate gene expression using shRNA libraries, and identify genes whose overexpression promote the MDS phenotype. Our ultimate endpoint is the detection of terminal differentiation and colony formation resulting in normal myeloid cells.

Hypothesis: Expression of a normal human cDNA library in MDS cells will correct the aberrant phenotype and permit normal proliferation and myeloid differentiation in selected clones.

Body

In this study we test the assumption that over-expression of a wild type gene, or suppression of an abnormally expressed gene by shRNA, in a transfected MDS clone will interrupt the pathogenic signaling that gives rise to the MDS phenotype. Therefore, the transfected gene will permit normal differentiation of the MDS cell into a normal myeloid lineage. The first objective (months 1 – 6 in SOW) was to prepare and characterize the lentivirus libraries.

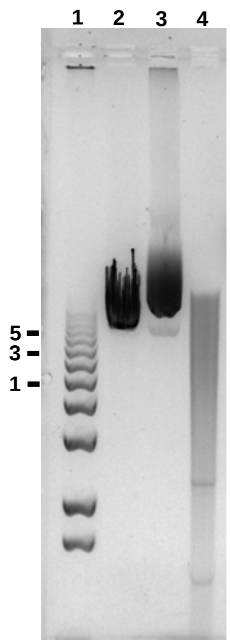


Fig. 1. Construction of human liver cDNA expression library. Library contains ~7 x 10^7 individual clones, and the average insert size is >2 kb. Lane 1: marker, size indicated in kb; Lane 2: linearized plasmid; Lane 3: plasmid and ligated insert; Lane 4: human liver cDNA insert prior to ligation.

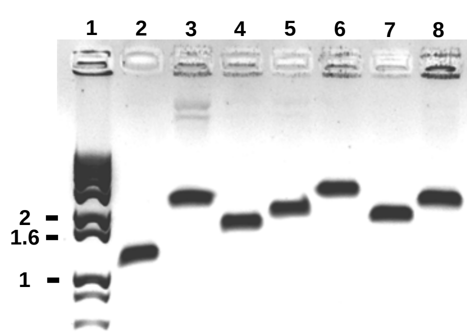


Fig. 2. Characterization of lentivirus cDNA expression library. Following packaging and titering of the lentivirus preparation, 293T cells were infected with library at multiplicity of infection of 0.1 and selected with puromycin x 1 wk. Individual surviving clones were expanded over 10 days and screened by PCR using LTR-specific primers. Lane 1: marker, size indicated in kb; Lanes 2 - 8: PCR product from individual clones.

Our next goal was to transfect human MDS cells with lentivirus, select for survivors with puromycin, and test for expression of cDNAs contained in the library. Surviving colonies were dissociated, plated as replicas, and aliquots were cryopreserved for future murine studies. Replated colonies were tested for their proliferative and differentiative response to GM-CSF (months 3 – 9 in SOW).

Fig. 3. Transfection of human MDS cells with lentivirus cDNA expression library. MDS bone marrow cells from 5q- (pictured at right), mono 7 / 7q-, trisomy 8, and del 20q were transfected at an moi of 0.1 with the lentivirus library described above. After 14 days growth in soft agar surviving colonies were harvested, dissociated, replated as replicates, and aliquots were cryopreserved. Cells transfected with empty lentivirus particles did not generate proliferating colonies. 200x

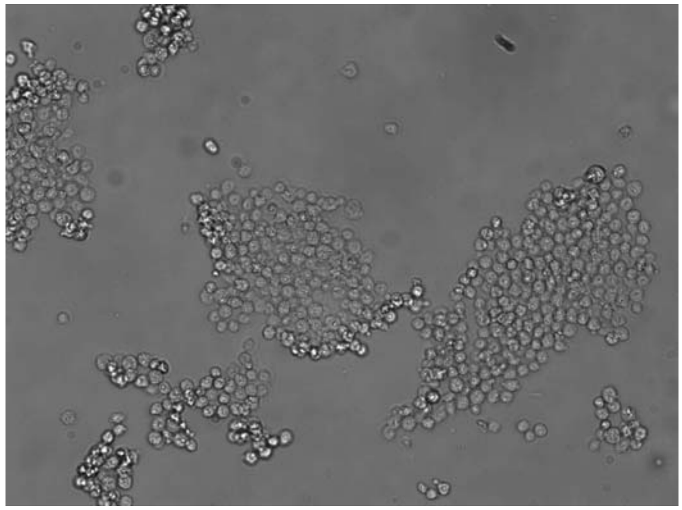


Fig. 4. Detection of lentivirus-encoded GFP in transfected human MDS cells. cDNA inserts encode GFP fusion proteins. Fluorescence microscopy demonstrated strong expression of GFP in puromycin-resistant surviving colonies. 200x

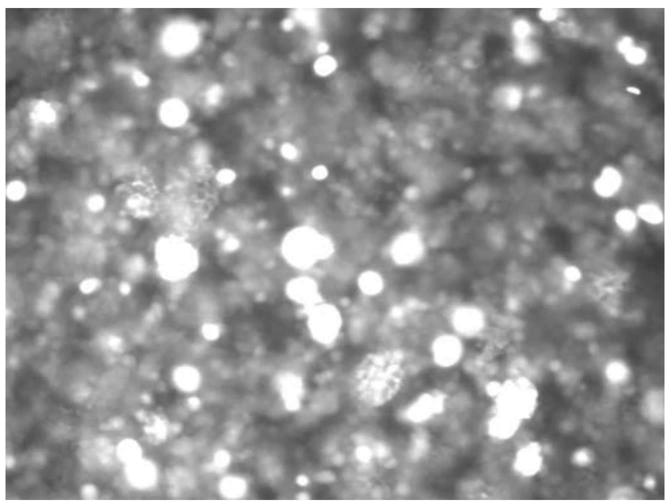
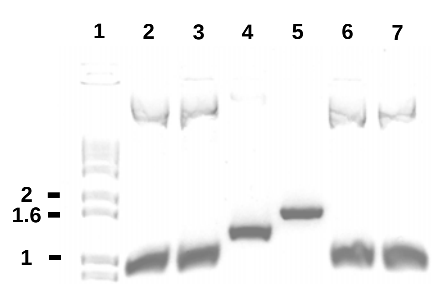


Fig. 5. Expression of lentivirus-encoded cDNA-GFP fusion proteins in transfected human MDS cells. Seven GFP-positive colonies were isolated and screened by PCR using LTR-specific primers to demonstrate that fluorescent MDS cells also retained expression of the encoded library-derived cDNA.



The next objective was to characterize surface markers and phenotype the successful transfectants (months 6 – 12 in SOW). Normal bone marrow cells have a low percentage of blasts (~2-3%), represented by gated population (Fig. 6, black circle). This gated population has low expression of c-kit and CD34 in normal marrow. MDS marrow with elevated blasts (lower panels) have high expression of c-kit and CD34. We are still in the process of evaluating c-kit and CD34 in our collection of lentivirus transfected MDS isolates.

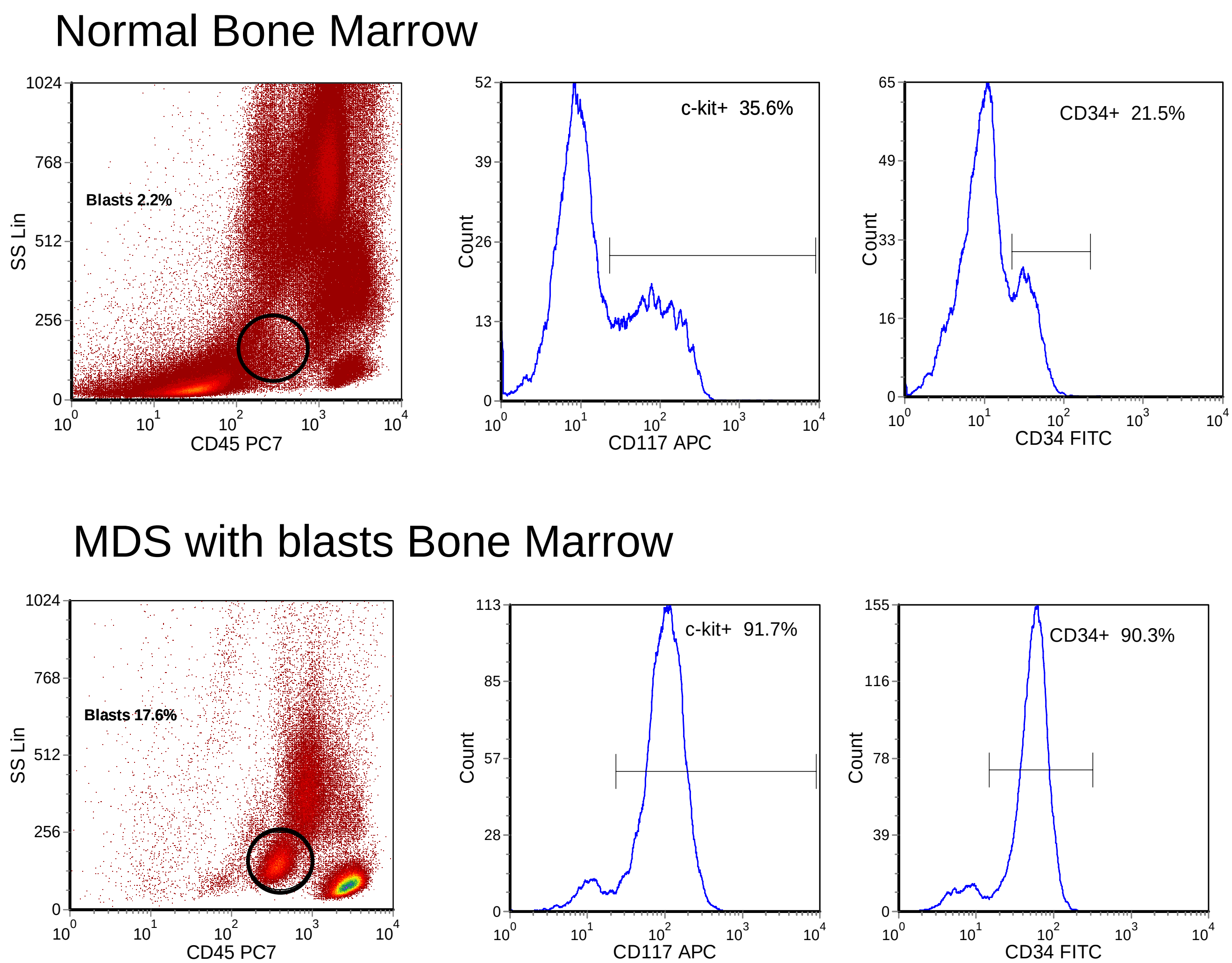


Fig. 6. Characterization of MDS surface markers. Normal human bone marrow (upper panels), and 7q- MDS marrow (lower panels) was stained for CD45 (leukocyte common antigen), CD34 (stem cell marker), and CD117 (c-kit).

We have not yet inoculated NOD.Cg-*Prkdcscid Il2rgtm1Wjl*/SzJ mice (NSG mice) (1-8) with successful transfectants expressing candidate gene. The last objective is to test ability of transfectants to engraft NSG mice and generate chimeric bone marrow (months 12 – 18). This objective will be the most difficult. There is possibility that NSG mice will not engraft well with transfected human MDS marrow. However, the recent development of NSG-SGM3 mice (Jackson Laboratories), in which NSG mice have been engineered to express human stem cell factor, GM-CSF, and IL-3, may provide additional trophic benefit for growth of human xenografted marrow (9). We have recently placed an order for these mice, and they should be arriving shortly.

Key Research Accomplishments

1. Creation of cDNA lentivirus libraries
2. Transfection of human MDS marrow
3. Expression of cDNA-GFP fusion proteins in MDS cells
4. Identification of cDNAs that drive proliferation in MDS cells
5. Characterization of markers associated with MDS blasts

Reportable Outcomes

This data was reported at the 2011 Case Comprehensive Cancer Center Retreat, Corporate College East, July 8, 2011.

Conclusion

We expect to identify several genes that when overexpressed, confer proliferative capability to MDS isolates. Some of these may be known cellular oncogenes; some may be genes that have not previously been known to promote growth; some may function to drive cells toward the AML phenotype. Identification of novel proliferative genes might shed light on new mechanisms of cell growth control and oncogenesis, and might be of therapeutic value if gene expression could be inhibited by an shRNA method in vivo. We expect to identify a number of marrow-expressed cDNAs that may overcome minimal defects that are present in some MDS clones and confer a phenotype in which transfected MDS clones exhibit gain of function and regain the ability to differentiate into functional myeloid cells and support successful engraftment of NSG mice. Identification of cDNAs that can successfully complement the MDS phenotype can be potentially investigated in future human gene therapy trials. Finally, we expect to isolate a smaller number of novel cDNAs, that are not normally expressed in the myeloid lineage, that will confer a normal differentiation phenotype through activation of alternate pathways that are not normally utilized in myeloid cells. Due to the unbiased, completely random nature of this complementation technique, and the underlying abnormal biochemical pathways at work in the MDS clones, it is unlikely that complementation will correct a majority of MDS defects and allow normal differentiation to proceed. However, even if only a handful of MDS phenotypes can be reversed out of a sample size of 300, it would permit greater understanding of the pathophysiology of the disease, and generate leads as to new potential therapies.

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